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14. ABSTRACT

Exposure to stress has been shown to increase tumor growth in a number of animal models in association with greater tumor angiogenesis and elevated vascular endothelial growth factor (VEGF), an important initiator of angiogenesis and tumor progression. Similarly, tumor cell lines *in vitro* are susceptible to β-adrenergic receptor (β-AR)-mediated elevation of VEGF and other proangiogenic and prometastatic factors, such as interleukin-6 (IL-6) and matrix metalloproteinases (MMP). Together, these results suggest that stress-induced activation of the sympathetic nervous system and norepinephrine (NE) release stimulates tumor cell β-AR to promote tumor growth at least in part by facilitating tumor angiogenesis. We previously determined that MDA-MB-231 (MB-231), a human breast cancer cell line that represents the more aggressive 'triple negative' phenotype, expresses many β-AR sites per tumor cell. By comparison, 4T1, a murine mammary adenocarcinoma cell line, expresses no detectable β-AR surface expression or signaling. To investigate a role for sympathetic nervous system involvement via β-AR stimulation in breast cancer pathogenesis *in vivo*, we have assessed sympathetic tyrosine-hydroxylase-positive (TH+) innervation of MB-231 and 4T1 tumors grown orthotopically (in the mammary fat pad). Furthermore, we investigated the impact of sympathetic activation on *in vivo* tumor growth of MB-231 and 4T1 tumors using two approaches to activate the sympathetic nervous system: 1) social isolation, a chronic stressor that has been shown to facilitate ovarian tumor growth; and 2) chronic desipramine treatment (a tricyclic antidepressant used clinically that inhibits NE reuptake).

15. SUBJECT TERMS
breast cancer, sympathetic nervous system, norepinephrine, angiogenesis, VEGF

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INTRODUCTION

Cancer patients often experience chronic emotional stress and/or other negative psychological factors, such as depression or lack of social support, with diagnosis and successive treatment. A recent clinical trial showed that reducing stress decreased cancer recurrence and related-death in breast cancer survivors [1]. Exposure to stress has been shown to influence tumor growth in a number of animal models [2]. Thaker and colleagues discovered that ovarian tumors from chronically stressed mice manifested a two-fold increase in growth in association with greater tumor angiogenesis and elevated vascular endothelial growth factor (VEGF), an important initiator of angiogenesis and tumor progression [3]. The stress effects were blocked *in vivo* by pharmacologically blocking β-adrenergic receptors (β-AR). Nasopharyngeal, ovarian, and breast carcinoma and melanoma cell lines *in vitro* are similarly susceptible to β-AR-mediated elevation of VEGF and other proangiogenic and prometastatic factors, such as interleukin-6 (IL-6) and matrix metalloproteinases (MMP) [4-7]. Hence, *we hypothesize that stress-induced activation of the sympathetic nervous system (SNS) and norepinephrine (NE) release stimulates tumor β-AR to augment breast tumor growth and metastasis by facilitating angiogenesis.* Therefore, we propose to examine the impact of sympathetic innervation on *in vivo* breast tumor growth and angiogenesis. We believe that therapeutic targeting of SNS signaling, using β-AR blockade, is a promising method for inhibition of stress-induced angiogenesis.

BODY

Task 1: Continue formal and informal education in oncology.

1a. Formal education in oncology. (18 months)

I attended and presented a poster at the Psychoneuroimmunology (PNI) Research Society conference in June 2011 where there was a National Cancer Institute-sponsored symposium titled, "PNI and Cancer: Role of the Tumor Macrophage Environment," which was very relevant to my research. I also attended and was a poster contestant finalist at the DoD BRCP Era of Hope conference in August 2011. I will attend an American Association for Cancer Research (AACR) meeting within the next year.

1b. Informal education in oncology. (ongoing)

I continue to participate in weekly lab meetings, where members of the lab present their research or papers about tumor biology. I also attend lectures relevant to my research, including talks hosted by the Breast Cancer Research Group (part of the University of Rochester's James P. Wilmot Cancer Center).

1c. Laboratory training. (ongoing)

I continue laboratory training pertinent to my project. I have recently begun using our two photon laser scanning microscope to analyze angiogenesis and to measure second harmonic generation.

1d. Clinical training. (ongoing)

I continue to participate in longitudinal clinical experiences, where I spend several hours per week with physician preceptors and their patients learning about clinical breast cancer. I have worked with a breast cancer oncologist, Dr. Michelle Shayne, and a geneticist, Dr. Chin-To Fong, who screens patients for BRCA mutations.

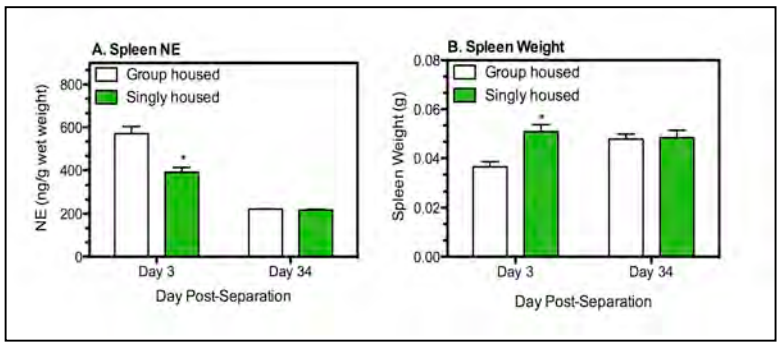
Task 2: Determine if chronic stressor exposure stimulates breast tumor growth and metastasis, and if the stress effect can be inhibited by β-adrenergic receptor (β-AR) blockade.
(Months 0-24)

Task 2a: Measure the catecholamine response to social isolation. (2-5 months)

We initially investigated the impact of stressor exposure, in the form of social isolation, on orthotopic growth of the high β-AR-expressing, human breast tumor cell line MB-231 in severe combined immunodeficiency (SCID) mice. Although an initial pilot experiment showed increased MB-231 growth in isolated mice, we found it difficult to replicate this finding (described in detail in Task 2b). In addition, tumor norepinephrine (NE) concentration was either not changed or not dramatically altered in the socially isolated mice. To understand how social isolation drives sympathetic activation, we measured NE concentration in the spleen, a highly-innervated organ, to assess peripheral changes in NE levels associated with social isolation. After adaptation to group housing, all SCID mice were injected with MB-231 cells in the mammary fat pad on day 0. When tumors were detectable in all mice (day 14 post-tumor injection, when tumor volumes were 25-50 mm^3), one-half of the mice were singly housed. The rest of the mice remained in their home cages. A subset of mice in each group was sacrificed 3 days post-separation to measure NE levels early after separation. At this time point, the tumors were too small to measure NE (≤10 mg).

Day 3 post-separation, splenic NE concentration was significantly reduced in the singly housed mice (Fig. 1A; ANOVA, housing x day interaction, $p < 0.001$). The decrease in NE concentration was associated with increased spleen weight at this time point (Fig. 1B; ANOVA, housing x day interaction, $p = 0.02$), suggesting that the increase in spleen mass reduced NE concentration in the singly housed mice. By day 34 post-separation, neither NE concentration nor spleen weight was altered in singly housed compared to group housed mice. Furthermore, compared to day 3, splenic NE concentration at day 34 was significantly reduced in group housed mice in conjunction with increased spleen mass (ANOVA, main effect of day, $p < 0.001$). In this experiment, it was not possible to measure tumor NE concentration at day 3, but tumor NE was not altered at day 34 (data not shown). These results demonstrate an early effect of social isolation, which suggests that the impact of single housing is greater early after separation, and homeostatic mechanisms mitigate the impact of social isolation over time. We will continue to investigate the kinetics of NE concentration in tumors with social isolation and desipramine treatment (described in Task 2b).

Fig. 1. Effect of social isolation (single housing) on spleen NE concentration and weight 3 days after transfer to single housing. Results are expressed as mean ± SEM. n=5 in both groups. Asterisk indicates different versus group housed at that time point by Neuman-Keuls post-hoc analysis.



Task 2b: Determine if chronic stressor exposure alters orthotopic tumor growth and metastasis in mice. (6-12 months)

When social isolation was begun 7 days prior to MB-231 injection, neither tumor growth (Fig. 2A) or tumor weight (Fig. 2B) were altered at the time of sacrifice (83 days after MB-231 injection). These experimental results are representative of three experimental repetitions. In this experimental repetition, we detected a small but significant increase in tumor NE concentration at the time of sacrifice (Fig. 2C; Student's t-test, $p = 0.03$). However, neither human (tumor-derived) or mouse vascular endothelial growth factor (VEGF) were altered in any repetition (data not shown). In this experimental repetition, human interleukin 6 (IL-6) was not altered (data not shown), but mouse IL-6 was significantly reduced in the singly housed mice (Fig. 2D; Student's t-test, $p = 0.03$), suggesting a potent effect of NE on the tumor stroma. We postulated that the inability to produce replicable changes in tumor growth was due to the fact that MB-231 is a slow-growing tumor *in vivo*, allowing mice to adapt to any early stress-induced alterations, as shown in Fig. 1.

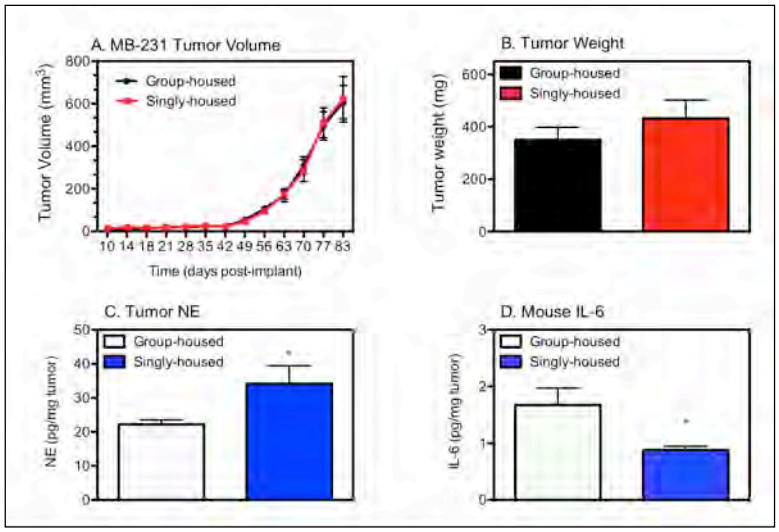


Fig. 2. Effect of social isolation 7 days prior to MB-231 injection. These figures represent three experimental repetitions. Results are expressed as mean ± SEM. n=9 group-housed, n=8 singly housed. Asterisk indicates significantly different vs group housed ($p < 0.05$ by Student's t-test).

With the social isolation-induced stressor, we realized that we were unable to produce a sustained stress response (Fig. 1) necessary for the many weeks required for MB-231 tumor growth *in vivo* (Fig. 2A). We found that increasing the number of tumor cells injected into the mammary fat pad did not greatly accelerate tumor growth. Therefore, we decided to revise our model in two ways: 1) we utilized the treatment desipramine (DMI), a NE-reuptake inhibitor used to treat patients suffering from depression, to elevate NE as a pharmacologic "stressor" exposure; and 2) we altered our *in vivo* tumor model to 4T1, a metastatic murine mammary adenocarcinoma cell line. 4T1 differs from MB-231 in several important ways: it is grown in immunocompetent BALB/c mice; the primary tumor grows rapidly in the mammary fat pad; and it quickly metastasizes to the lung from the primary tumor. We found that 4T1 cells express no detectable β-AR and do not respond to β-AR agonists with elevated cAMP. We view 4T1 as a good model for examining NE effects on the tumor stromal cells in the absence of direct involvement of β-AR-expressing tumor cells.

Fig. 3A shows 4T1 tumor growth over the course of 19 days in BALB/c mice implanted subcutaneously with either placebo or 21-day release, 5 mg DMI pellets. 4T1 cells were injected into the mammary fat pad 2 days after implantation of the pellets. There was a trend toward increased tumor volume in the DMI-treated group at days 12 and 14 (Student's t-test, $p = 0.081$ and $p = 0.073$, respectively), but this effect dissipated by the end of the experiment. At the time of sacrifice, there was similarly a trend toward increased tumor weight (Fig. 3B; Student's t-test, $p = 0.12$) and tumor NE concentration (Fig. 3C; Student's t-test, $p = 0.24$). The small increase in tumor growth suggested that a higher dose of DMI may be more effective.

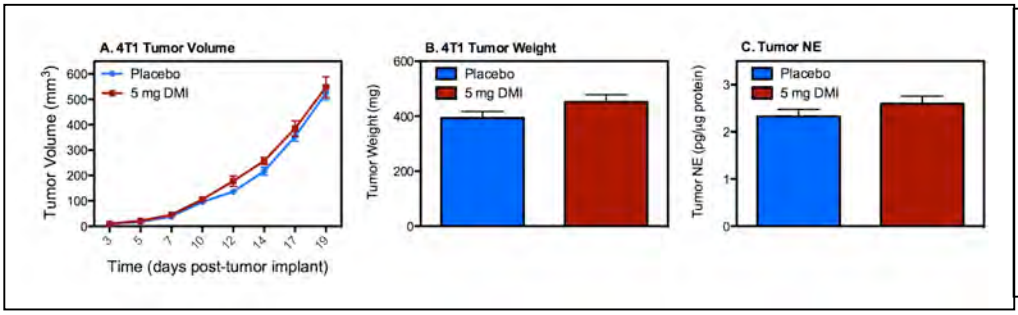


Fig. 3. Effect of low dose DMI on 4T1 tumors. Results are expressed as mean ± SEM. n=10 placebo, n=9 DMI.

In Fig. 4, we repeated this experiment with 21-day release, 10 mg DMI pellets. Tumor volume was significantly increased in the DMI-treated group on days 12 and 14 (Fig. 4A; repeated measures ANOVA, main effect of treatment $p = 0.047$; Bonferroni's post-hoc analysis $p < 0.05$ and $p < 0.001$, at day 12 and 14, respectively). Mice were sacrificed on day 14, and tumor weight was likewise increased (Fig. 3B; Student's t-test, $p = 0.031$). Fig. 3C shows that tumor NE concentration was not altered by the treatment, but spleen NE concentration was almost double in the DMI-treated group (Fig. 4E; Student's t-test, $p = 0.010$) in the absence of difference in spleen weight (Fig. 4D; Student's t-test, $p = 0.17$), confirming that our treatment elevates NE peripherally.

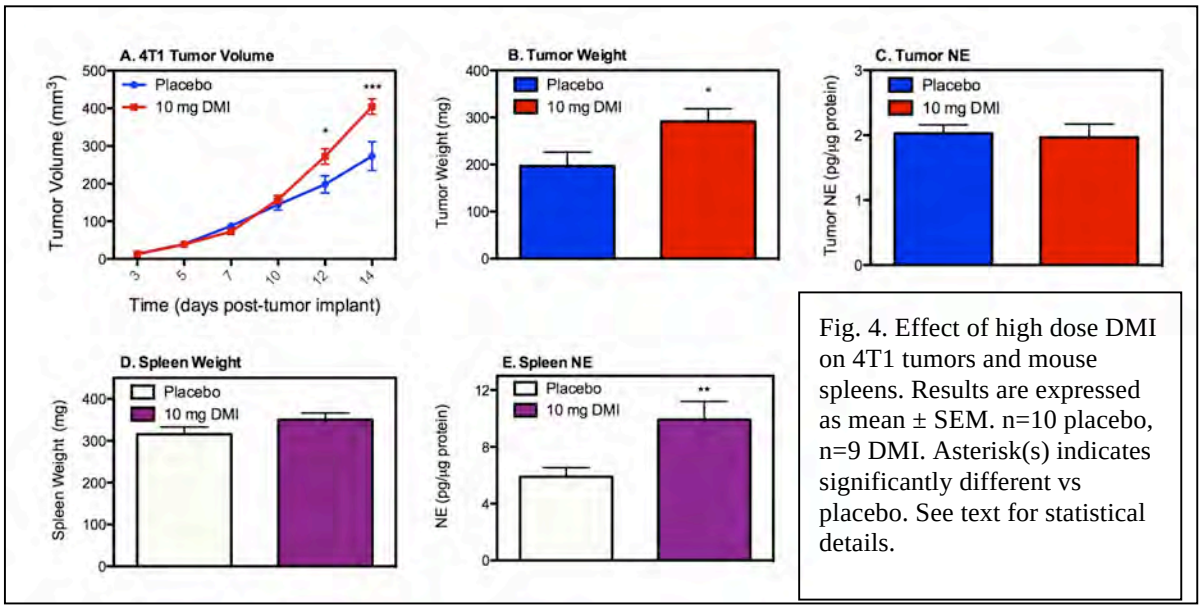


Fig. 4. Effect of high dose DMI on 4T1 tumors and mouse spleens. Results are expressed as mean ± SEM. n=10 placebo, n=9 DMI. Asterisk(s) indicates significantly different vs placebo. See text for statistical details.

We further examined the tumors for the proangiogenic or prometastatic cytokines vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and matrix metalloproteinase-9 (MMP-9) and the lungs for metastasis (Fig. 5). There was a trend toward reduced IL-6 (Fig. 5B; Student's t-test, $p = 0.012$) but no difference in MMP-9 (Fig. 5C; Student's t-test, $p = 0.30$) or lung metastasis (Fig. 5D; Student's t-test, $p = 0.61$). There was a significant decrease in VEGF (Fig. 5A; Student's t-test, $p = 0.019$). This decrease was unanticipated because we expected a DMI-induced increase in tumor volume would be due to increased VEGF.

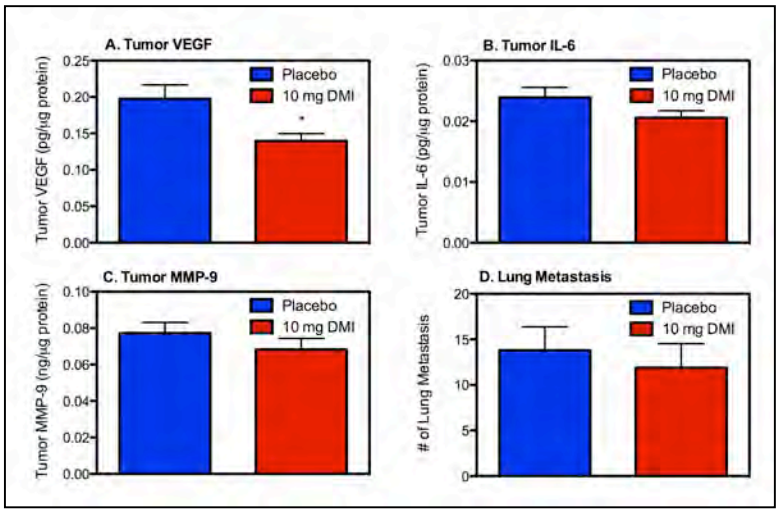


Fig. 5. Effect of high dose DMI on 4T1 tumor cytokine concentrations and lung metastasis 14 days after tumor injection. These results are from the same mice shown in Fig. 4. Results are expressed as mean ± SEM. n=10 placebo, n=9 DMI. Asterisk indicates significantly different vs placebo ($p < 0.05$ by Student's t-test).

To determine if a DMI-induced increase in VEGF was elevated prior to increased experimental tumor growth, we performed this same experiment as a time course: mice were sacrificed on days 7 and 12 after tumor injection. Fig. 6 shows that on day 7, there was no difference in tumor weight (Fig. 6A; Student's t-test, $p = 0.09$), VEGF concentration (Fig. 6B; Student's t-test, $p = 0.25$), or IL-6 (Fig. 6C; Student's t-test, $p = 0.78$). In the treatment group on day 12, there was a trend toward increased tumor weight (Fig. 6D; Student's t-test, $p = 0.066$) and decreased tumor IL-6 (Fig. 6F; Student's t-test, $p = 0.056$). As in the previous experiment that ended on day 14, there was a significant decrease in tumor VEGF (Fig. 6E; Student's t-test, $p = 0.026$) on day 12 when there was an increase in tumor growth. Therefore, no increase in VEGF was detected at earlier time points when tumor weights were equivalent. We are currently quantifying blood vessel density to determine if angiogenesis is altered with DMI treatment.

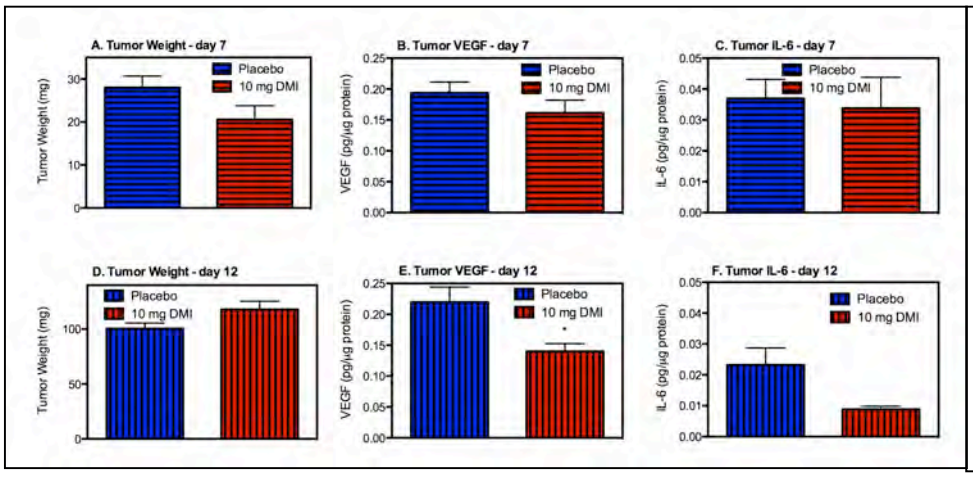


Fig. 6. Effect of high dose DMI on 4T1 tumor weight, VEGF, and IL-6 after 7 or 12 days of growth *in vivo*. Results are expressed as mean ± SEM. n=9 placebo, n=6-7 DMI. Asterisk indicates significantly different vs placebo ($p < 0.05$ by Student's t-test).

To determine if NE was altered, we measured spleen NE concentration on day 7 (Fig. 7B). Of note, the spleens from the DMI-treated group were significantly greater in weight (Fig. 7A; Student's t-test, $p < 0.0001$), similar to the effect of social isolation on spleen weight (Fig. 1). On day 12, there was no difference in spleen weight (Fig. 7C; Student's t-test, $p = 0.21$) or spleen NE concentration (Fig. 7D; Student's t-test, $p = 0.96$). This result was not consistent with the previous experiment where there was increased spleen NE after DMI treatment (Fig. 4E) and needs to be repeated, including NE measured in the tumors.

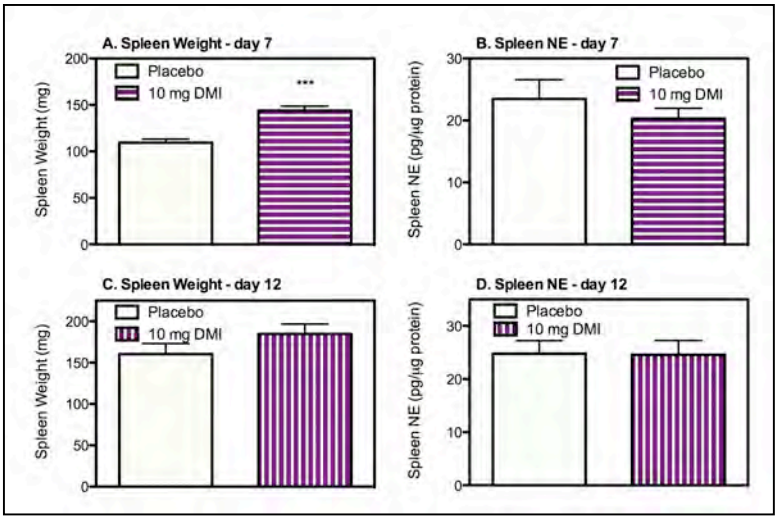


Fig. 7. Effect of high dose DMI on the spleens of 4T1-tumor-bearing mice after 7 or 12 days of tumor growth. These are from the same mice shown in Fig. 6. Results are expressed as mean ± SEM. n=9 placebo, n=6-7 DMI. Asterisks indicate significantly different vs placebo ($p < 0.0001$ by Student's t-test).

Task 2c: Determine if chronic stress-induced alterations in breast cancer growth *in vivo* can be blocked by a clinically available β-AR antagonist. (6-12 months)

Due to the inability to show a consistent elevation in stress-induced tumor growth in the previous parts of Task 2, we did not test β-AR blockade in the context of stress. We have begun preliminary experiments to determine if β-AR blockade prevents the effects of DMI. We have unexpectedly encountered a high mortality rate when β-blockade is combined with the 10 mg DMI pellet. In a pilot study, we have determined that 7.5 mg DMI combined with the β-blocker nadolol is well tolerated. We will now determine the impact of treatment of 7.5 mg DMI pellets combined with β-AR blockade.

Task 3: Determine if breast cancer metastasis to the brain increases with chronic stressor exposure and, further, if it can be inhibited by β-AR blockade.
(Months 8-20)

We have no progress to report on this task.

Task 4: Determine if chronic stressor exposure alters the ordering of collagen in tumors as quantified with second harmonic generation (SHG).
(Months 20-32)

I have begun the training necessary to use the two photon laser scanning microscope to measure SHG in the context of the DMI experiments, but there is currently no experimental progress to report.

Task 5: Examine sympathetic innervation, tumor and non-tumor cell types expressing β-AR, and collagen ordering in human breast tumors.
(Months 24-30)

The purpose of this aim was to determine which tumor and stromal cells express β-AR, the anatomical distribution of tyrosine hydroxylase (TH+) sympathetic nerve fibers within the tumor, and the relationship between β-AR expression, TH+ nerves, and collagen ordering in paraffin-embedded tumor biopsies from breast cancer patients. Though we have not performed this in human samples yet, Fig. 8 shows fluorescent staining of orthotopic MB-231 (Fig. 8A-B) and 4T1 (Fig. 8C-D) tumors for tyrosine hydroxylase (TH)+ noradrenergic nerve fibers and CD31+ blood vessel endothelial cells. TH+ nerves are more prevalent in the periphery of the tumor, where they are often associated with blood vessels.

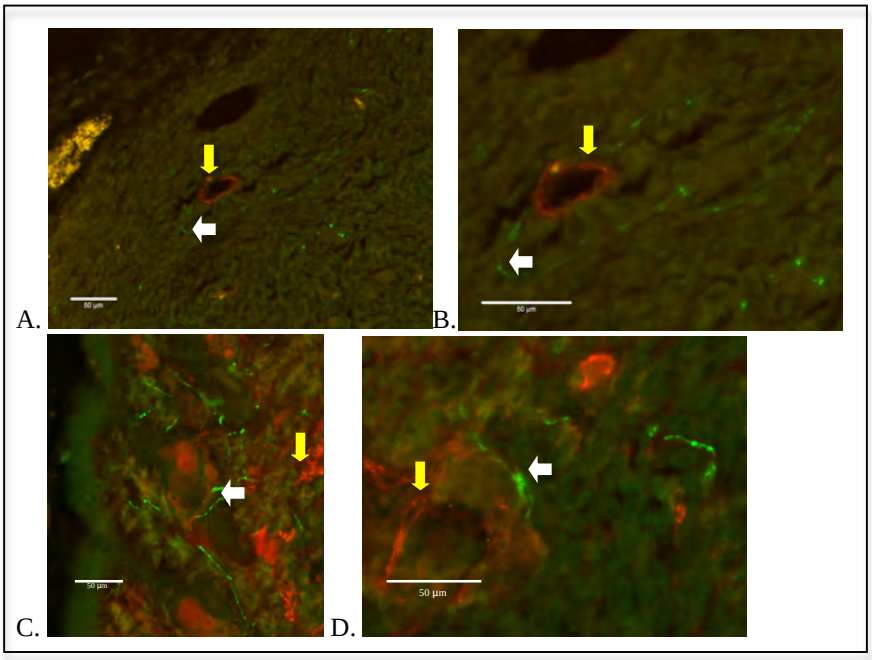


Fig. 8. Sympathetic innervation and blood vessels in MB-231 and 4T1 tumors. TH+ noradrenergic nerve fibers (green – white arrows) and CD31+ endothelial cells (red – yellow arrows) in MB-231 (A-B) and 4T1 (C-D).

KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that the impact of social isolation is of limited duration. This may be of particular significance when a tumor is slow-growing.

- Using 4T1, an aggressive breast tumor model that does not express detectable β-AR, we demonstrated that NE elevates tumor growth, suggesting that tumor stromal cell components may facilitate tumor cell growth, even when the tumor is not able to directly respond to NE.

- The mechanism(s) mediating the desipramine-induced increase in tumor growth is not increased VEGF and IL-6 production, key drivers of angiogenesis, as we hypothesized, suggesting alternative mechanisms may elicit primary tumor growth upon stressor exposure and activation of the sympathetic nervous system.

REPORTABLE OUTCOMES

Poster Presentations:

Evidence for Sympathetic Nervous System and Norepinephine Regulation of Breast Cancer Pathogenesis. **Szpunar MJ**, Madden KS, Liverpool KM, Brown EB. 15th Annual University of Rochester Cancer Center Symposium. Nov. 11, 2010. Rochester, NY.

Sympathetic Nervous System Innervation and Function in a Beta-Adrenergic Receptor Negative Breast Cancer Model. **Szpunar MJ**, Madden KS, Liverpool KM, Brown EB. Psychoneuroimmunology Research Society Annual Meeting. June 8-11, 2011. Chicago, IL.

Szpunar MJ, Madden KS, Liverpool KJ, Brown EB. Sympathetic nervous system innvervation and function in breast cancer models. Department of Defense Breast Cancer Research Program Era of Hope Meeting. August 2-5, 2011. Orlando, FL.

Publication:

Madden KS**, Szpunar MJ**, Brown EB. 2011. β-Adrenergic Receptors (β-AR) regulate VEGF and IL-6 production by divergent pathways in high β-AR-expressing breast cancer cell lines. *Breast Cancer Res Treat.* Epub Jan 14, 2011.

CONCLUSION

We had predicted that *in vivo* growth of MB-231, a high β-AR-expressing breast cancer cell line, would be sensitive to sympathetic nervous system activation via social isolation, a stressor known to activate the sympathetic nervous system. We have been unable to establish a consistent effect of social isolation on MB-231 growth. The results presented here suggest that 1) the timing of stressor exposure relative breast tumor growth may be an important determinant of the impact of stressor exposure on breast tumor growth, and 2) that adaptation to stressor exposure may minimize the impact of a stressor such as social isolation, particularly when a tumor is relatively slow-growing, such as MB-231 in SCID mice.

The murine breast cancer model 4T1 is useful for characterizing sympathetic nervous system-induced alterations in breast tumor growth in the absence of tumor β-AR signaling. Chronic DMI treatment elevated NE and increased 4T1 tumor growth, but we found no evidence that increased tumor growth was mediated through increased tumor angiogenesis, as has been observed with high β-AR-expressing cancer cells. DMI may elicit increased angiogenesis through other angiogenic regulators. Alternatively, other systems that regulate tumor growth, such as the immune system, may be responsible for altered tumor growth.

Our results imply that the impact of sympathetic nervous system activation and β-AR stimulation on breast cancer pathogenesis varies with tumor cell β-AR expression and signaling capability. Furthermore, the impact of sympathetic activation and subsequent response to stressor exposure is

modulated by the ability of the host to adapt to the activation and elevated NE. Understanding the implications of heterogeneity of tumor cell β-AR expression in the context of stressor exposure is critical to predict efficacy of therapeutics that block or activate the sympathetic nervous system, including drugs commonly used clinically for other conditions, including cardiovascular disease and anti-depression therapies.

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APPENDICES

N/A